The Role of Oxidative Processes in Emphysema[1-3]

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SUMMARY Elastase/elastase inhibitor imbalance in the lung has been implicated in the pathogenesis of pulmonary emphysema. In light of this, it may be significant that the activity of two major elastase inhibitors, alpha 1-proteinase inhibitor (alpha 1-antitrypsin, α1Pi) and bronchial mucous proteinase inhibitor, can be decreased by oxidizing agents. The effect can be observed with ozone, substances present in cigarette smoke, and oxygen metabolites generated by lung macrophages as well as peroxidative systems released by other phagocytic cells. Thus α1Pi recovered from lung washings of cigarette smokers has only half the predicted normal activity per mg inhibitor and contains 4 moles of methionine sulfoxide (oxidized methionine) per mole of inactive inhibitor. By contrast, α1Pi purified from nonsmokers' lung washings is fully active and contains only native methionine. At the same time, lung washes from some smokers show significantly greater hydrolytic activity against a specific synthetic elastase substrate than do lung washes of nonsmokers. These findings suggest that some smokers may develop an acquired imbalance between elastase and elastase inhibitor in their lungs, favoring activity of the enzyme. In addition to the potential effect of cigarette smoking on lung elastase/elastase inhibitor balance, smoking also may interfere with elastin repair mechanisms. Specifically, acidic water-soluble gas phase components of cigarette smoke prevent synthesis of desmosine cross-links during elastinogenesis *in vitro*. This report will attempt to correlate the foregoing information on biochemical changes in the lung induced by cigarette smoking with the development of emphysema in the smoker.

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Introduction

The theme of this symposium is "Proteases and Antiproteases in the Lung," especially the potential importance of these substances in mediating various forms of lung injury. The subject has been reviewed elsewhere, particularly in connection with the pathogenesis of pulmonary emphysema (1-6). It is well known that oxidizing agents also can produce tissue damage; indeed both proteases and activated species of oxygen have been implicated as mediators of immunologic lung injury (7). This report attempts to link these two pathways of pulmonary connective tissue destruction because, in addition to their capacity to injure cells and intercellular matrix directly, oxidants can also perturb the homeostatic balance between proteases and their regulatory inhibitors (8, 9). Particular emphasis will be placed on the inactivation of alpha 1-proteinase inhibitor (the major regulator of neutrophil elastase in the lower respiratory tract of man (10)) by oxidants in cigarette smoke and in secretions of phagocytic cells. Several other actions of cigarette smoke, which may also contribute to connective tissue abnormalities in the lung, will be discussed. Possible biochemical links between pulmonary emphysema and cigarette smoking will be summarized and several questions raised for future study.

Mechanism of Elastase Inhibition By Alpha 1-Proteinase Inhibitor and the Effect of Oxidizing Agents

Alpha 1-proteinase inhibitor (alpha 1-antitrypsin, α1Pi) appears to act as a pseudosubstrate for its target proteases, and the site at which recognition and/or proteolysis occurs represents the active-site of the inhibitor (11). Structural studies have shown that the active site of α1Pi contains a methionine-serine peptide bond (12-14), although the possible presence of multiple active sites has not been ruled out (14, 15). Apparently this methionine-serine bond is recognized early in a series of reactions that ultimately leads to formation of extremely stable complexes between one mole of protease and one mole of inhibitor. The high stability of this complex may be based on covalent bonding between the protease's active center and the active site of the inhibitor, although the precise mechanism is not known. In any event, the inhibition of elastase by α1Pi depends on the integrity of a methionine-serine sequence in the active site of the inhibitor, and perhaps that of an additional methionine residue near the active site as well (12, 13, 16). In addition to methionine, the configuration of lysine (17) and tyrosine (18) residues in α1Pi may also affect its antiprotease activity.

Oxidation of α1Pi by chloramine-T or N-chlorosuccinimide, under conditions that favor the selective oxidation of methionine thioether groups, results in a loss of inhibitory activity (13, 16, 19-22). Structural studies have since confirmed that inactivation of α1Pi by chloramine-T or N-chlorosuccinimide is associated with the oxidation of two methionine residues in the inhibitor, one of which is present in the methionyl-seryl active site (13, 22). Oxidation of the active site methionine residue results in reduced interaction of the inhibitor with neutrophil and pancreatic elastase as well as trypsin (13, 20-22). Chymotrypsin-inhibitory capacity of α1Pi is relatively unaffected by oxidation, whereas elastase-inhibitory activity is most severely affected (13, 20-22).

Oxidation of the inhibitor reduces the rate of its association with neutrophil elastase by a factor of 2,000 compared with that of the native inhibitor (21). Any elastase-α1Pi complexes that may be formed appear to be less stable than normal (21). Similar findings have been obtained with synthetic elastase substrates containing methionine adjacent to the scissile bond. Oxidation of the methionine side-chain in these synthetic peptides also reduces the rate of their interaction with PMN elastase (19, 23). Suppression of the inhibitory capacity of α1Pi by oxidation also renders this protein susceptible to proteolytic digestion. After incubation of oxidized α1Pi with elastase, the inhibitor is partially degraded (21). In addition to methionine oxidation, a recent study has shown that specific oxidation of tyrosine residues in α1Pi can also inactivate its inhibitory function (18).

Besides α1Pi, a potent local inhibitor of neutrophil elastase and cathepsin G is found in bronchial secretions (24). The bronchial mucous inhibitor (BMPi) has been purified and shown to be a cationic protein with a molecular weight of 10 to 13,000 daltons (25, 26). Gadek and colleagues (10) reported undetectable levels of BMPi in bronchoalveolar lavage fluids of humans, and they and others (27) have suggested that the primary function of BMPi is to protect the upper airways (bronchi, trachea, and nasopharynx) against proteolytically-mediated injury. The active site structure of BMPi is not presently known; however, oxidizable residues also appear to be present close to the reactive center of this inhibitor (Fritz H, personal communication). A closely related,

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low molecular weight, acid-stable inhibitor in dog submandibularis glands contains a methionyl-aspartyl bond in its elastase and chymotrypsin-directed reactive center (28). As might be expected, BMPi can also be inactivated by chemical oxidants *in vitro* (29).

To assess the potential importance of these findings for the pathogenesis of pulmonary emphysema in humans, one need only note that when the activity of circulating α1Pi is depleted by treatment of experimental animals with a chemical oxidant, connective tissue changes suggestive of early stages of panacinar emphysema develop (30). The human respiratory tract is constantly exposed to various sources of oxidizing activity. In addition to oxygen itself, selected human populations are exposed to oxidizing environmental pollutants such as ozone, oxides of nitrogen and sulfur, and a host of oxidizing free-radicals present in cigarette smoke (see following). Moreover, phagocytic cells present in the lung (alveolar macrophages, neutrophils) generate copious amounts of hydrogen peroxide and other activated oxygen metabolites (see following), and this effect is increased by smoking. In the following section, some of these oxidants and their *in vitro* and *in vivo* effects on lung elastase-inhibitors will be discussed in further detail.

Oxidants Produced by Combustion of Cigarettes

Cigarette smoke displays a wide range of free-radical mediated activities including peroxidation and alkylation reactions both *in vitro* and *in vivo* (31). Aqueous solutions of cigarette smoke can oxidize cysteine sulfhydryl groups, a reaction that is blocked by the free-radical scavenger thymol (32). Cigarette smoke contains approximately 2×10^{15} free radicals per puff (33). Half of these appear to be polymeric phenoxy radicals related to melanin-like tobacco leaf pigments and are present in the particle fraction of cigarette smoke. They have a half-life of 10 hours on cigarette filters or in organic solvents after extraction of filters. An equal number of free radicals are found in the gas phase of cigarette smoke. These are mainly oxygen-centered radicals with half-lives of 10 min in the gas phase and 1 min in aqueous solution. In addition to the radicals produced during burning of cigarettes, cigarette smoke also may contain up to 800 ppm of nitrogen oxides (NO_x), especially NO. Besides their own free-radical properties, NO_x can react with olefins in the gas phase of cigarette smoke (34) or with peroxides (including, perhaps, the H_2O_2 produced by lung macrophages) to generate potent oxy-radicals such as hydroxyl radical (34, 35).

In Vitro *Inactivation of Antiproteases by Cigarette Smoke Solutions*

Freshly prepared aqueous solutions of unfractionated cigarette smoke, partly clarified by low-speed centrifugation, can suppress the elastase-inhibitory capacity of human serum (19). Immunoelectrophoresis of mixtures of aqueous smoke solution, human serum, and elastase show decreased elastase-α1Pi complexes and increased free, active protease. At least in our hands, smoke-treated α1Pi and control α1Pi are indistinguishable with respect to total quantity, electrophoretic mobility, and antigenic behavior of the inhibitors. An important finding is that free radical scavengers such as thymol or hydroquinone prevent the suppression of serum elastase-inhibition by cigarette smoke (19). In the presence of such agents, elastase-α1Pi complexes again can be detected following immunoelectrophoretic analysis of enzyme-smoke-serum reaction mixtures, and free enzyme is not seen. Also, treatment of serum with model oxidants under conditions that favor selective oxidation of methionine groups leads to a similar suppression of serum elastase inhibition (16, 19, 20). These observations suggest that oxidation reactions, perhaps mediated by smoke radicals of the type discussed in the preceding section, may be responsible for the observed results.

Similar findings have recently been obtained using ozone, in place of cigarette smoke, as a source of oxidizing activity (36). Ozone decreased the inhibition of trypsin, chymotrypsin, and elastase by human α1Pi, both in plasma and in solutions of the pure inhibitor. Total loss of pancreatic elastase inhibitory activity required 18 moles of ozone per mole of pure α1Pi. (A corresponding loss of the ability to inhibit human leukocyte elastase also was observed). Ozone-inactivated α1Pi was reported to contain four residues of methionine sulfoxide as well as oxidized tyrosine and tryptophan residues (36).

Inactivation of the bronchial mucous inhibitor (BMPi) *in vitro* by aqueous cigarette smoke solutions (29) or tar fractions (37) also has been demonstrated. Thin-layer gel-immunofiltration analysis of mixtures of aqueous smoke-treated BMPi and human leukocyte elastase revealed decreased elastase-BMPi complexes, increased uncomplexed BMPi, and increased free elastase (29). Phenolic antioxidants also prevented the suppression of the elastase-inhibiting-capacity of BMPi by aqueous cigarette smoke (29). In addition, treatment of BMPi with chemical oxidants caused a similar suppression of elastase-inhibition (29).

Oxidants Produced by Phagocyte Metabolism

The oxidizing systems of phagocytes, which participate in microbial-killing by these cells, have recently been reviewed in some detail (38, 39). According to present knowledge, the main features of oxygen metabolism in polymorphonuclear neutrophil leukocytes (PMN) and, with some modification, in monocytes and macrophages are as follows.

Upon phagocytosis or exposure to certain membrane-active agents, phagocytes undergo a "respiratory burst" characterized by increased oxygen consumption, increased production of hydrogen peroxide, and increased utilization of glucose via the hexose monophosphate shunt. The increase in oxygen consumption may be related to activation of a cyanide-insensitive, membrane-bound, NAD(P)H-dependent oxidoreductase that catalyzes the single electron reduction of molecular oxygen to the superoxide radical, as in equation (i):

$$2O_2 + NAD(P)H \rightarrow 2O_2^{\cdot} + NAD(P)^{+} + H^{+}.$$

The NAD(P) generated in this reaction stimulates the hexose monophosphate shunt because NAD(P) is a substrate in the first step of this metabolic pathway. In this first step, glucose-6-phosphate is converted to 6-phosphogluconate by glucose-6-phosphate dehydrogenase. At the same time, NAD(P) is converted to NAD(P)H, thus replenishing the substrate required for the membrane oxidase in equation (i). NAD(P)H generated by the shunt is also utilized by glutathione reductase. (The latter enzyme, together with glutathione peroxidase, functions to detoxify any excess hydrogen peroxide produced by PMN in the course of the "respiratory burst".)

The superoxide radical formed in equation (i) can react directly, or this radical can generate more powerful activated oxygen species (*) or oxygen free radicals (**) by the reactions shown in equations (ii) and (iii).

Equation (ii), dismutation reaction:

$$O_2^{\cdot} + O_2^{\cdot} + 2H^{+} \rightarrow H_2O_2^{*} + O_2$$
$$\text{(or singlet } O_2?)^{*}$$

Equation (iii), Haber-Weiss type reaction, trace metal (lactoferrin (40)?) catalyzed:

$$O_2^{\cdot} + H_2O_2 \rightarrow OH^{\cdot **} + OH^{-} + O_2$$
$$\text{(or singlet } O_2?)^{*}$$

Theoretically, these activated oxygen species and oxygen-free radicals could inactivate α1Pi within the phagocytic vacuole or in the immediate environment of the phagocytic cell.

In addition to the reaction shown in equation (iii), hydrogen peroxide can participate in other oxidative processes by serving as a cofactor for myeloperoxidase of PMN and monocytes. A second cofactor, usually a halide such as chloride or iodide anion, also is required. (Thyroid hormones can serve as a source of iodide for PMN.) In these myeloperoxidase-catalyzed reactions, hypohalous acids (e.g., HOCl) or free halogens (e.g., iodine) can be formed and these, in turn, are powerful oxidizing agents.

Activation of the membrane-oxidase that initiates the foregoing series of reactions (see equation i) appears to require a limited-proteolysis step mediated by a serine-

enzyme with chymotrypsin-like activity (41). However, PMN and monocytes of individuals with chronic granulomatous disease appear incapable of activating the membrane-oxidase during phagocytosis or exposure to membrane-active agents. In these subjects, a normal "respiratory burst" does not accompany the stimulatory event. This condition is one of several in which phagocyte oxidative reactions are impaired, with serious consequences for host antimicrobial defense.

Enhanced Oxidant Production by Lung Macrophages of Cigarette Smokers

Chronic cigarette smoking not only increases the numbers of PMN (42) and macrophages (43) in the lung, but also activates these cells metabolically (43). Among the metabolic pathways that are stimulated in smokers' macrophages are those leading to production of superoxide radical and H_2O_2 (44). It therefore seems reasonable to propose that stimulated phagocytes in the lungs of cigarette smokers might be capable of generating oxidants that, in turn, could inactivate proteinase inhibitors in the environment of these cells. Evidence showing that stimulated phagocytes can indeed inactivate α1Pi and BMPi *in vitro* has been obtained in several laboratories; some of this evidence will now be summarized.

In Vitro *Inactivation of Antiproteases by Products of Oxygen Metabolism in Phagocytes*

We previously showed that human PMN, phagocytosing opsonized antigen-antibody complexes absorbed to the outer surface of dialysis membranes, produce diffusable species of activated oxygen capable of inactivating the elastase-inhibitory capacity (EIC) of whole serum or pure α1Pi on the other side of the membrane (45). In this system, serum EIC was partially protected by superoxide dismutase, catalase, or mannitol, suggesting that hydroxyl radical, formed by interaction of superoxide radical and hydrogen peroxide, might have been responsible for the observed inactivation. Sodium azide also partly protected EIC, implicating myeloperoxidase-mediated reactions as well. A cell-free superoxide generating system (xanthine + xanthine oxidase) also was shown to inactivate α1Pi in this system.

In related studies, Matheson and colleagues (46, 47) demonstrated that purified myeloperoxidase (MPO) in the presence of hydrogen peroxide and a halide ion could catalytically inactivate α1Pi *in vitro*, whereas two protease inhibitors more resistant to oxidants (α2 macroglobulin and α1-antichymotrypsin) were not affected by such treatment. These same investigators also directly showed that the active site methionine residue in α_1Pi, as well as an adjacent methionine residue, had been oxidized to the sulfoxide form by the MPO system (47).

More recently, we have extended our studies of phagocyte-mediated oxidative inactivation of α1Pi to include human mononuclear phagocytes (blood monocytes and alveolar macrophages), as well as PMN (48). These cells were activated by a selective membrane perturbing agent, phorbol myristate acetate (PMA), rather than by phagocytosis. Under these conditions, "respiratory burst" products were formed without bulk release of azurophil granule constituents. Such stimulated cells generated superoxide radical and suppressed elastase-inhibition by human serum. Scavengers of superoxide radical and H_2O_2 partly prevented this effect. Immunoelectrophoretic analysis of elastase reacted with serum that had been exposed to stimulated PMN showed decreased α1Pi-elastase complexes and free elastase. Treatment of phagocyte-inactivated serum with dithiothreitol (a reducing agent) resulted in significant recovery of inhibitory activity against elastase suggesting that, originally, α1Pi had been oxidatively inactivated. Moreover, PMN and monocytes from a patient with chronic granulomatous disease (which do not undergo a "respiratory burst"), as expected, failed to produce detectable levels of superoxide anion after stimulation with PMA, and these cells also failed to suppress serum elastase-inhibition.

Although PMA stimulates phagocyte respiration (including O_2^- and H_2O_2 production), this agent induces only minimal azurophil degranulation. However, because myeloperoxidase (MPO) is capable of catalytically inactivating α1Pi (one mole of purified MPO was shown to inactivate 250 moles of α1Pi per min, in a cell-free system (46)), the small quantities of this enzyme released following PMA stimulation could combine with H_2O_2 generated by the "respiratory burst" and extracellular halide and could participate in α1Pi-oxidation in our experiments. In support of this, we were able to demonstrate that azide and cyanide (both inhibitors of heme enzymes such as MPO) partly protected elastase-inhibiting capacity of serum against inactivation by the phagocytes. Furthermore, depletion of Cl^- from the medium also partly protected serum elastase inhibitory capacity from inactivation by stimulated PMN, suggesting that inactivation of α1Pi is partly dependent on a halide cofactor. Catalase, which decreases available H_2O_2, almost completely protected serum EIC from inactivation by PMA-stimulated cells.

In addition to the foregoing studies, Clark and coworkers (49) also reported that the MPO-system plays an important role in mediating oxidative inactivation of α1Pi by PMA-stimulated PMN, but they found a less significant inhibition by O_2^- and its derivative-activated oxygen species. Although mature human macrophages are believed not to contain myeloperoxidase, macrophages may contain other forms of peroxidase. Indeed, recent evidence suggests that a macrophage peroxidatic enzyme also may be able to inactivate α1Pi in a cell-free system (47).

The prediction that α1Pi may be inactivated by phagocyte-derived oxidizing systems *in vivo* has been borne out by the work of Wong and Travis (50), who demonstrated inactive α1Pi in synovial fluid obtained from inflamed joints and showed that the inactive inhibitor was in the oxidized state. A total of four methionine residues per molecule, including the active site methionine, were present as methionine sulfoxide. Other amino acids were unmodified, although a 17 amino acid peptide fragment was missing from the amino terminus of the inhibitor.*

The low molecular weight mucous inhibitor, human BMPi (that helps protect the mucus-lined airways against PMN elastase and cathepsin G), also has been shown to lose activity after *in vitro* exposure to myeloperoxidase, hydrogen peroxide, and chloride ion (29). Tsan and Chen (51) recently even demonstrated oxidation of free methionine by PMN and ascribed the effect to myeloperoxidase-mediated reactions and to singlet oxygen.

Macrophage H_2O_2 Also Could Act Synergistically with Oxidants in Cigarette Smoke to Inactivate Lung α1Pi

Pryor and coworkers (35) have shown that NO (and its derivative NO_2) can react with H_2O_2 to produce hydroxyl radical (a potent oxidizing agent) and other oxy-radicals, and have suggested that such reactions could take place in the lungs of smokers (34). Presumably, metabolically stimulated lung macrophages could serve as a source of H_2O_2 (see preceding section on enhancement of macrophage H_2O_2 production in cigarette smokers), whereas NO_x have been identified as cigarette combustion products. Oxy-radicals produced by interactions of H_2O_2 and No_x could contribute to the oxidative inactivation of lung α1Pi in the cigarette smoker (34). Evidence indirectly supporting this suggestion has been obtained by Cohen (personal communication),† who finds that aqueous smoke solutions from

* Since this article was submitted, Campbell and coworkers studied neutrophils in contact with ^{125}I-labeled fibronectin and reported that oxidants released from the stimulated cells did not protect secreted elastase against inhibition by α1Pi. (Campbell EJ, Senior RM, McDonald JA, Cox DL. Proteolysis by neutrophils. Relative importance of cell-substrate contact and oxidative inactivation of proteinase inhibitors in vitro. J Clin Invest 1982; 70:845–52.)

† Also, Cohen AB, James HL. Reduction of the elastase inhibitory capacity of alpha 1-antitrypsin by peroxides in cigarette smoke. An analysis of brands and filters. Am Rev Respir Dis 1982; 126:25–30.

different brands of cigarettes inactivate α1Pi in direct proportion to their ability to oxidize o-dianisidine, *and that addition of H_2O_2 to the smoke solutions greatly augments both o-dianisidine oxidation and α1Pi inactivation*. Cohen also suggests that H_2O_2 produced by macrophages in the small airways of cigarette smokers could enhance the oxidative damage to lung α1Pi caused by constituents of inhaled cigarette smoke.

Thus multiple reaction pathways can be envisioned by which α1Pi could be oxidized and inactivated in the lungs of smokers. Direct action of oxidants in smoke is one possibility. Another is that the involved oxidants are produced by lung cells stimulated by smoke exposure. Still a third could involve oxidants generated by interactions between smoke components and products of cell metabolism. It now remains to present the evidence supporting the view that oxidative inactivation of α1Pi actually takes place *in vivo*.

In Vivo *Evidence for Inactivation of Lung Antiproteases by Cigarette Smoking*

Earlier, we found that brief inhalation exposure of rats to 3 or 6 puffs of cigarette smoke significantly decreased elastase inhibitory capacity per mg α1Pi in lung lavage fluid (52). Gadek and colleagues measured the inhibitory activity of α1Pi obtained from the lungs of smoking and nonsmoking humans and reported a nearly twofold reduction in the functional activity (versus pancreatic elastase) of this inhibitor in the lungs of cigarette smokers (53). Recently, we confirmed the fact that functional activity of α1Pi is significantly decreased (40%) in smokers' bronchoalveolar lavage fluids (BAL) compared with α1Pi derived from nonsmokers' BAL. In our study (54), the suppressant effect of smoking on α1Pi activity was shown using PMN elastase as test enzyme. Moreover, suppression of α1Pi was found to be reproducible when selected smokers and control individuals were lavaged on repeated occasions. Crossed antigen-antibody electrophoresis using antibodies directed against α1Pi and elastase confirmed that inactivation of α1Pi was responsible for the decrease in EIC of smoker's BAL. However, other workers (Stone and coworkers, Aspen Lung Conference, June 1982) failed to detect decreased levels of functional α1Pi in the lower respiratory tract of human smokers. A critical variable may be the length of elapsed time between last cigarette smoked and lung lavage in different laboratories.

Cigarette smoking also may be associated with decreased functional activity of bronchial mucous proteinase inhibitor (BMPi). Starting with tracheal aspirates obtained from patients undergoing elective surgery for non-pulmonary disorders, we partially purified BMPi from mucus collected from 15 smokers and 21 nonsmokers. Trypsin, chymotrypsin, and PMN elastase-inhibiting-capacities of the partly purified fractions were measured. In comparison to nonsmokers, smokers showed a 20% decrease in functional activity of BMPi per mg of inhibitor (measured by radial immunodiffusion against anti-BMPi antibody). This reduction was not related to patient age or sex (29).

Evidence that In Vivo *Inactivation of α1Pi in Cigarette Smokers is Due to Oxidation*

Using the animal model referred to in the preceding section (i.e., rats acutely exposed to cigarette smoke inhalation), we obtained indirect evidence suggesting that lung α1Pi inactivation *in vivo* is caused by oxidation of the inhibitor. We found that rats adapted to oxidant-injury by exposure to 2 ppm ozone for 4 h, one wk prior to cigarette smoke inhalation, had normal levels of α1Pi activity in their lung lavage fluids after smoking. Control, unadapted rats showed a 30% decrease in elastase-inhibiting-capacity per mg lung α1Pi after identical smoke exposure (52). In addition, when lavage fluids obtained from smoke-exposed rats were dialyzed against the reducing agent, sodium metabisulfite, there was a 75% recovery of normal elastase-inhibiting-capacity (52). The recovered inhibitory capacity was solely due to reactivated α1Pi, because antibody to rat α1Pi completely abolished the activity of sodium metabisulfite-treated samples. The reducing agent had no effect on the activity of the test enzyme.

In studies on human smokers (54), we obtained direct evidence to support the suggestion that lung α1Pi inactivation *in vivo* is caused by oxidation of the inhibitor. It was shown that partially inactivated α1Pi purified from smokers' bronchoalveolar lavage fluids (BAL) contained methionine sulfoxide, whereas fully active inhibitor purified from BAL or serum of nonsmokers did not. (Although we only detected changes in methionine, our conditions were designed to facilitate methionine sulfoxide quantitation and, conceivably, may have masked additional changes in other amino acid residues). Four moles of methionine sulfoxide were detected per mole of inactive α1Pi, which conforms closely to the value obtained by Wong and Travis (50) for α1Pi purified from rheumatoid synovial fluid. In the latter case, sequence analysis showed that the active-site methionine had been oxidized. Similar studies will be necessary to establish the location of the oxidized methionine residues present in smokers' lung fluid α1Pi.

The partially inactive α1Pi present in smokers' BAL could not be distinguished from the active α1Pi present in nonsmokers' BAL on the basis of immunoreactivity, electrophoretic mobility, or molecular weight. Thus, smokers' lung α1Pi appears not to be grossly denatured, inactivated by complex formation with granulocyte proteases (55), or significantly degraded by thiol-proteases (56), or by macrophage elastase (57). These latter agents alter the native electrophoretic mobility (55) or molecular weight of the inhibitor (56, 57). Alpha 1Pi inactivation by cigarette smoke *in vivo* also is not likely due to nonspecific denaturing effects of smoke on lung proteins (37) because functional activity of α-1-antichymotrypsin (a protease inhibitor that is resistant to inactivation by oxidation [58]) is unchanged in smokers' BAL.

Does Cigarette Smoke also Inactivate PMN Elastase?

In order for the oxidation hypothesis of smoker's emphysema, as put forward previously, to have merit, it is also necessary to show that cigarette smoke does not inactivate leukocyte elastase to the same extent as it does α1Pi. Although the enzyme is known to be more resistant than the inhibitor to inactivation by chemical and phagocyte-derived oxidants (20, 29, 45, 48), the relative rates of inactivation of these two proteins by *cigarette smoke* are unknown. Recently, Ohlsson and colleagues (37) reported that human granulocyte elastase is severely inactivated by dimethylsulfoxide solutions of the tar fraction of cigarette smoke, and Ejiofor and coworkers (59) reported partial inactivation of the enzyme by water-soluble, gas phase components of cigarette smoke. In the latter study, the enzyme was incubated with a relatively high concentration of smoke solution for prolonged time periods and the rate of its inactivation was measured. However, no effort was made to compare this rate with that of α1Pi-inactivation under identical incubation conditions.

We recently carried out an experiment (60) to compare the inactivation of human serum α1Pi and human granulocyte elastase by different concentrations and types of smoke extracts. Various amounts of aqueous solutions of gas phase cigarette smoke were incubated with pure human leukocyte elastase or with crude human leukocyte granule extract for 10 min at 37° C, and the effects on enzyme activity determined using a synthetic amide substrate. Simultaneously, equal concentrations of the same smoke solutions were incubated with 10% human serum under identical conditions, and the effects on serum inhibition of purified or crude leukocyte elastase were similarly measured.

In separate experiments, aqueous solutions of unfractionated cigarette smoke were incubated with pure leukocyte elastase or with serum for 30 min at 22° C. Ability of the smoke-treated enzyme to digest elastin and of the smoke-treated serum to inhibit elastin-digestion was then determined by measuring radial elastolysis in elastin-agar plates. Both experimental protocols clearly showed that serum elastase-inhibiting capacity is much more susceptible to inactivation by cigarette smoke than is leuko-

cyte elastase when these proteins are incubated with water-soluble smoke extracts for relatively short times. For example, at concentrations of smoke that inactivated half the α1Pi (mimicking observed levels of α1Pi activity in smokers' lung wash (53, 54), only a 10% loss in PMN elastase activity could be measured. Similar results were obtained with both synthetic (amide) and natural (elastin) substrates, gas phase smoke and unfractionated whole smoke, and with pure enzyme and crude leukocyte granule extract (60).

An obviously important question is whether a short or a long incubation time *in vitro* best duplicates the conditions governing interactions among elastase, α1Pi and inhaled smoke *in vivo*. Our results at least suggest that should α1Pi and elastase in lung fluids be simultaneously exposed to inhaled smoke, half the antiprotease could become inactivated before the enzyme was significantly affected. This would potentiate the ability of elastase to attack its substrates in the lung before its own activity was suppressed by smoke products.

Other Effects of Cigarette Smoking on Lung Protease-Antiprotease Balance

Although not immediately relevant to the present discussion of oxidative processes in emphysema, at least brief mention should be made of the fact that cigarette smoking also causes increased numbers of PMN and macrophages to appear in the lung (42, 43), both of which cell types have the capacity to secrete elastase. The recruitment of PMN into smokers' lungs may involve prior activation of pulmonary macrophages by smoke components because stimulated alveolar macrophages can elaborate chemoattractants for PMN (42, 61–64). Further details can be found in earlier reviews of the subject (e.g., 5, 43).

The increased numbers of lung macrophages and PMN caused by smoking (as well as the inactivation of elastase inhibitors) leads to the prediction that increased elastase activity might be detectable in lung fluids of smokers. In preliminary studies by the authors (Janoff A, Raju L, Dearing R, unpublished observations), 7 out of 11 smokers showed elevations in PMN elastase activity of lung fluids compared with 11 nonsmoking control subjects (albeit involving very small amounts of enzyme (< 10 ng/mg lavage albumin)). The enzyme was measured in concentrated bronchoalveolar lavage effluent using the highly sensitive and specific elastase substrate, butyloxycarbonyl-l-alanyl-l-alanyl-l-prolyl-l-valyl-aminomethylcoumarin (65) (a generous gift of Dr. Morris Zimmerman; Merck Institute, Rahway, NJ). In one experiment, activity was completely inhibited by phenylmethanesulfonyl fluoride (10 mM) but only partly inhibited by ethylenediaminetetraacetate (10 mM), suggesting that the elastase might be a serine enzyme (PMN or monocyte-derived) rather than a metallo-enzyme (macrophage-derived) or that both enzymes were present. It may have been complexed to α2-macroglobulin because, in another experiment, activity of lavage was not significantly decreased by added serum.

Thus, lung fluids of smokers appear to contain some oxidized, inactivated α1Pi (see previously) and, in some cases, small amounts of elastase activity (but more than is found in nonsmokers' lungs). Taken together, these findings point to the possibility that protease-antiprotease balance in the lung may well be disturbed by such diverse effects of smoking as recruitment and stimulation of protease-secreting cells and interference with protease-inhibiting functions of α1Pi and BMPi. In the next section, an additional, newly discovered effect of smoking on elastin synthesis will be described, suggesting that even a third "chemical lesion" may result from smoking; one that could further exacerbate the development of emphysema in susceptible individuals.

Does Cigarette Smoking Exert an Inhibitory Effect on Elastin Repair?

Desmosine is a major cross-linking amino acid in elastin, and its formation is dependent upon the oxidative deamination of ϵ-amino groups of lysine residues in tropoelastin by the copper-dependent enzyme, lysyl oxidase. We recently (66) measured desmosine formation during elastinogenesis *in vitro*, using a cell-free system consisting of tropoelastin purified from chick embryo thoracic aorta or porcine aorta and lysyl oxidase purified from chick embryo or bovine lung. Desmosine formation was measured quantitatively using a specific, highly sensitive radioimmunoassay previously described (67). Desmosine synthesis could be demonstrated in this system with both homologous components (chick tropoelastin and chick lysyl oxidase) as well as with heterologous components (pig tropoelastin and bovine enzyme). Synthesis could be inhibited 80 to 90% in either system in the presence of a 1:20 dilution of aqueous extract of fresh cigarette smoke. Moreover, the inhibitory component(s) also could be demonstrated in the gas phase of filtered cigarette smoke and they behaved as negatively charged substance(s) during ion-exchange chromatography (66).

More recently, we also showed that water-soluble components of the gas phase of filtered cigarette smoke inhibit the first step in lysyl oxidase-catalyzed oxidative deamination of free ϵ-amino groups of elastin. The inhibitory effect of smoke was dose dependent. Because this step is also involved in formation of other elastin cross-links besides desmosine, the inhibitory action of cigarette smoke may have broad effects on elastin synthesis. The foregoing studies are described in further detail elsewhere (68).

Independently, Osman and associates (69) have simultaneously reported that tobacco smoke exposure retards elastin repair *in vivo*. Using hamsters in which experimental emphysema was first induced by a single intratracheal injection of pancreatic elastase, these investigators showed that pulmonary elastin content is regained within 1 wk after injury in animals not exposed to cigarette smoke. By contrast, elastase-injured "hamsters exposed to 6 to 7 puffs of cigarette smoke at hourly intervals for 8 h/day during the same repair period do not resynthesize elastin at the same rate." These workers injected ^{14}C-lysine during the repair phase after injury and found that "smoke exposure reduced ^{14}C-lysine incorporation into desmosine and isodesmosine by more than 40 percent" (69).

It has been suggested that elastin resynthesis may limit the degree of anatomic deformity in pulmonary emphysema (70), largely on the basis of evidence showing that lysyl oxidase inhibitors (such as β-aminoproprionitrile) greatly exacerbate the lesions induced by intrapulmonary elastase administration (70). Thus, the demonstration that elastin-synthesis is blocked by tobacco smoke components (66, 68, 69), may also help to explain the increased risk of pulmonary emphysema associated with cigarette smoking.

A Final Speculation: Inhibition of Transglutaminase by Cigarette Smoke—Potential Pathophysiological Effects on Lung

Cigarette smoke contains a complex array of chemicals, and smoke solutions may therefore modulate other pathways of lung metabolism besides those already mentioned in this report. For example, lung transglutaminases may be inhibited by cigarette smoke because transglutaminase in plasma (activated factor XIII of the clotting cascade) recently was found to be suppressed by smoke solutions (71).

Transglutaminases catalyze several acyl transfer reactions in which γ-carboxamide groups of peptide-bound glutamine residues serve as acyl donors. Epsilon-(γglutamyl) lysine cross-link formation (as in cross-linking of fibrin polymers during hemostasis) is perhaps the best recognized example (72). However, tissue transglutaminases may have several other functions. For example, a transglutaminase in macrophages has been implicated in regulating receptor-mediated endocytosis of α2-macroglobulin and other molecules (73). Might suppression of such an enzyme by cigarette smoke affect the endocytic activity of pulmonary macrophages? Again, fibroblast transglutaminases have been implicated in stabilization of a rigidly cross-linked cytoskeleton conducive to a nonproliferative state (74). Might inhibition of this enzyme function by inhaled cigarette smoke derange cytoskeletal architecture in some lung cells and increase the risk of lung cancer in smokers?

Conclusions

I. Biochemical Links Between Cigarette Smoking and Pulmonary Emphysema—Current Status of the Hypothesis

Currently, three separate actions of cigarette smoking on lung metabolism have been recognized, which may act together to increase the risk of chronic obstructive lung disease in smokers. First, cigarette smoking may promote the oxidative inactivation of major elastase inhibitors in the lung (α1Pi and BMPi). Evidence was presented in this report implicating actions of smoke oxidants and of oxidants generated by metabolically-stimulated lung cells in smokers. Synergistic effects involving both forms of oxidizing activity also are possible. Second, smoking may simultaneously increase the elastase "burden" on the pulmonary connective tissue by activating the recruitment of macrophages and neutrophils to the lung. Third, newer evidence suggests a previously unsuspected effect of smoking, namely, inhibition of cross-link formation during resynthesis (repair) of elastin. These relationships are summarized in figure 1.

It seems reasonable to suggest that the distribution within the acinus of these three smoking-linked "biochemical lesions" (oxidation of α1Pi, recruitment of elastase-secreting cells, inhibition of elastin repair), might parallel the acinar distribution of inhaled smoke. Noxious components of cigarette smoke (oxidizing free radicals, cell-stimulating particulates, putative lysyl-oxidase inhibitors such as cyanide (75), or copper-chelators (76)) would be expected to have their greatest impact at the bifurcation of the terminal bronchiole and first-generation respiratory bronchioles (77). It is just in this centriacinar zone that the anatomic lesion of emphysema typically manifests itself in cigarette smokers. These relationships are depicted schematically in figure 2.

II. Questions for Future Study

Although the mechanisms proposed in this report, taken together, constitute an attractive hypothesis for the pathogenesis of emphysema in cigarette smokers, many questions still remain. Foremost among these is the need to explain the variable susceptibility among smokers to development of the disease. It seems safe to assume that there are other genetic and/or environmental risk factors affecting the outcome, in addition to those described previously.

One suggestion holds that antioxidants may play a key role. It has been suggested that ceruloplasmin, a major plasma antioxidant in man, can protect α1Pi against inactivation by myeloperoxidase (Taylor JC, personal communication). In addition, a newly discovered enzyme, methionine-sulfoxide reductase, is especially interesting in this regard. This enzyme was first purified from *E. coli*, but an analogous enzyme has since been demonstrated in mammalian tissues including lung and blood. The microbial enzyme can reduce methionine sulfoxide in proteins to native methionine (78) and is capable of partially reactivating chemically-oxidized α1Pi (79). Further studies of the mammalian methionine-sulfoxide reductase and of ceruloplasmin appear warranted because individual variation in the activity of these enzymes in lung or blood could conceivably exert a profound influence on pulmonary protease-antiprotease balance in smokers.

It is also worth noting that α1Pi function is depressed in bronchopulmonary secretions of infants exposed to hyperoxia, as part of current management protocols for infantile respiratory distress syndrome (IRDS) (80). Could protease-antiprotease imbalance, thus acquired, play a role in the development of bronchopulmonary dysplasia, a not infrequent, emphysema-like com-

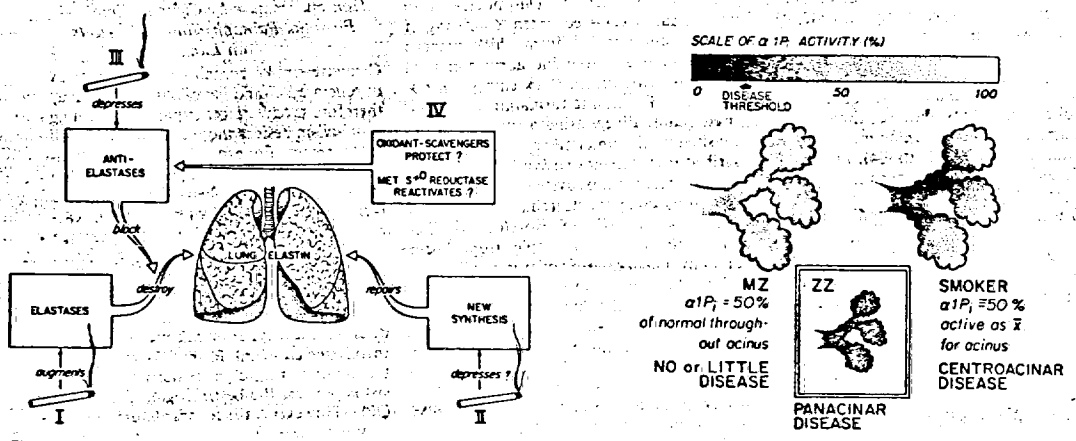

Fig. 1 (*left*). Proposed biochemical links between cigarette smoking and the pathogenesis of emphysema. (I) Smoking recruits monocytes, macrophages, and (through macrophage chemotactic factors) PMN to lung, elevating the connective tissue "burden" of elastolytic serine and metalloproteases. (III) At the same time, oxidants in smoke plus those produced by smoke-stimulated lung phagocytes (as well as oxidizing products of chemical interactions between these two) inactivate BMPi and α_1Pi, the latter representing the major antielastase "shield" of the respiratory units. (II) Other, unidentified water-soluble, gas-phase components of cigarette smoke (cyanide? copper-chelators?) inhibit lysyl oxidase-catalyzed oxidative deamination of ϵ-amino groups in tropoelastin and block formation of desmosine (and presumably other) cross-links during elastin synthesis, thus decreasing connective tissue repair. (IV) Anti-oxidants (ceruloplasmin? methionine-sulfoxide- reductase?) may protect or reactivate elastase-inhibitors, and other unidentified factors may modulate the chemical lesions induced in the lung by smoking so as to influence the risk of developing chronic obstructive lung disease. (Reproduced by permission from IM—Internal Medicine for the Specialist, Vol. 3, No. 3 March 1982.) Fig. 2 (*right*). Biochemical lesions induced by smoking are believed to parallel the distribution of inhaled smoke. In the diagram, only the inactivation of α_1Pi by oxidizing activity is shown, but the same reasoning can be extended to include the other 2 chemical lesions shown in figure 1. On the right-hand side of figure, the inhaled smoke constituents are shown as achieving their greatest concentration in the centriacinar zone (bifurcation of terminal bronchiole and first-generation respiratory bronchioles). Therefore, although the overall inactivation of α_1Pi measured in fluids recovered from the lower respiratory tract of smokers is about 50% (*see text*), the respiratory epithelium in the centriacinar structures may be more severely depleted of active elastase inhibitor. Thus, in smokers, active α_1Pi concentration in this zone may reach the disease threshold that is present throughout the acinus in homozygous α_1Pi deficiency (ZZ in the diagram). This could explain why centriacinar emphysema is most often associated with cigarette smoking whereas panacinar emphysema is more characteristic of ZZ disease. (MZ = heterozygous α_1Pi deficiency.) (Reproduced by permission from IM—Internal Medicine for the Specialist, Vol. 3, No. 3 March 1982.)

plication of IRDS? Similarly, oxidative processes may disrupt protease-antiprotease balance in the lungs of adult patients treated with hyperoxia for acute respiratory distress syndrome (ARDS). Abnormal functional levels of PMN elastase and α_1Pi have been reported in some ARDS patients (see Cochrane's report in this symposium).

Future work also may extend the oxidation hypothesis to embrace lung injury associated with exposure to other environmental pollutants besides cigarette smoke. For example, oxidizing gases such as the oxides of sulfur and nitrogen, as well as ozone, are frequent environmental pollutants in urban areas and industrial sites, and one of these (ozone) has already been shown to inactivate α_1Pi *in vitro* (36). However, direct oxidant-injury to lung connective tissue, independent of protease-mediators, also must be considered in all of the foregoing. Indeed, oxidant cytotoxicity (81–83) and oxidant-mediated damage to intercellular macromolecules such as hyaluronic acid (84, 85) and collagen (86) are well established. Direct injury by oxidants has been proposed as the chief mechanism responsible for the interstitial pulmonary fibrosis that can be induced in experimental animals by intratracheal instillation of peroxidases together with an H_2O_2-generating system (87). Similarly, the role of direct oxidant-mediated injury needs to be explored in the pathogenesis of emphysema of cigarette smokers and individuals exposed to industrial pollutants.

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